The dependence receptor DCC requires lipid raft localization for cell death signaling

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***DCC* (deleted in colorectal cancer) is a putative tumor suppressor gene whose expression is lost in numerous cancers. DCC also encodes the main receptor for the neuronal navigation cue netrin-1. It has been shown that DCC belongs to the so-called family of dependence receptors. Such receptors induce apoptosis when their ligand is absent, thus conferring a state of cellular dependence on ligand availability. We recently proposed that DCC is a tumor suppressor because it induces the death of tumor cells that grow in settings of ligand unavailability. Moreover, it seems that the DCC/netrin-1 pair may also regulate neuron survival during nervous system development. However, the mechanisms by which DCC triggers cell death are still unknown. We show here that the localization of DCC to lipid rafts is a prerequisite for its proapoptotic activity, both in immortalized cells and in primary neurons. The presence of DCC in lipid rafts probably allows the formation of an adequate submembrane complex, because the interaction of caspase-9 with DCC is inhibited by the disorganization of lipid rafts. Thus, dependence receptors may require lipid raft localization for cell death signaling.**

apoptosis | caspase | netrin | palmitoylation

Vogelstein and colleagues (1, 2) have shown that the development of colonic carcinoma from normal colonic epithelium is associated with the mutation of a specific set of genes. Allelic deletions [loss of heterozygosity (LOH)] on chromosome 18q in >70% of primary colorectal tumors prompted the search for a tumor suppressor gene at that locus. This search led to the cloning of a gene encoding a putative cell-surface receptor, *DCC* (deleted in colorectal cancer) (2). DCC expression was then shown to be markedly reduced in >50% of colorectal tumors. Moreover, the loss of DCC expression is not restricted to colon carcinoma but has been observed in many other tumors (for a review, see ref. 3).

DCC encodes an ≈200-kDa type I membrane protein of 1,447 aa, carrying an extracellular domain that displays homology to cell adhesion molecules (4). This homology has suggested that DCC may play a role in cell–cell or cell–matrix interactions. However, DCC-mediated cell aggregation has not been firmly established (3). Tessier-Lavigne and coworkers (5, 6) have demonstrated that DCC functions as a component of a receptor complex that mediates the effects of the axonal chemoattractant netrin-1. The role of DCC in mediating growth cone extension has been supported by the analysis of DCC-knockout mice, which display abnormal brain development (7).

However, the link between the putative role of DCC as a tumor suppressor and its ability to bind netrin-1 and mediate axon guidance was not clear until we proposed that DCC is a dependence receptor (8). DCC is functionally related to other dependence receptors such as $p75^{NTR}$, the androgen receptor, RET, Ptc, UNC5H, and neogenin (9–13). Such receptors create cellular states of dependence on their respective ligands by inducing apoptosis when unoccupied but inhibiting apoptosis in the presence of their respective ligands (13). We have shown that the expression of DCC induces apoptosis in the absence of netrin-1, whereas the presence of netrin-1 blocks this proapoptotic activity.

The fact that DCC displays such proapoptotic activity when not bound by its ligand has led to the hypothesis that the netrin-1/DCC pair may regulate tumorigenesis: Indeed, DCC can kill tumor cells that grow in an inappropriate context (e.g., local growth in a setting of constant and limited netrin-1 concentration or growth at a secondary site where there is no netrin-1 expression). Along this line, mice overexpressing netrin-1 in the gut show a marked decrease of cell death in the intestinal epithelium, which is associated with an increased predisposition to develop colorectal tumors (14). Thus, DCC may be viewed as a tumor suppressor that controls tumorigenesis by regulating apoptosis (15). In a very different instance, this dependence effect may be of crucial importance for the development of the nervous system. Netrin-1 knockout mice not only show neuronal navigation problems but also display increased cell death, as shown in the developing brainstem (16). Moreover, we have shown not only that netrin-1 controls commissural axon guidance but that it is also required to maintain the life of commissural neurons by inhibiting DCC-induced cell death (unpublished work).

However, the mechanisms that direct/control DCC-induced cell death are still unclear. DCC was demonstrated to be a caspase substrate with the major site of cleavage at D1290. The caspase cleavage of DCC was shown to be required for the proapoptotic effect, which is equivalent to that which has been shown for other dependence receptors (13). Not only is DCC cleaved by a caspase, but we have also shown that DCC induces apoptosis in a caspase-9-dependent pathway, albeit by a mechanism that is independent of the intrinsic (mitochondria-dependent) apoptotic pathway. We have also shown that DCC recruits caspase-3 and caspase-9, resulting in the activation of caspase-3 by caspase-9. In this regard, DCC defines an alternative pathway for apoptosis induction (17).

Because we and others recently observed that a fraction of DCC is constitutively associated with cholesterol- and sphingolipid-enriched membrane domains called lipid rafts and that this association is important for netrin-1-mediated axon guidance (18, 19), we wondered whether this particular raft localization may also be of importance for the other facet of DCC, apoptosis induction. Here we show that this raft localization is a prerequisite for the proapoptotic activity of DCC, both *in vitro* and in primary com-

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Abbreviations: DRM, detergent-resistant membrane; CO, cholesterol oxidase; CTB, cholera toxin subunit B; SMase, sphingomyelinase; HA, hemagglutinin epitope; HRP, horseradish peroxidase; AFC, 7-amino-4-trifluoromethylcoumarin.

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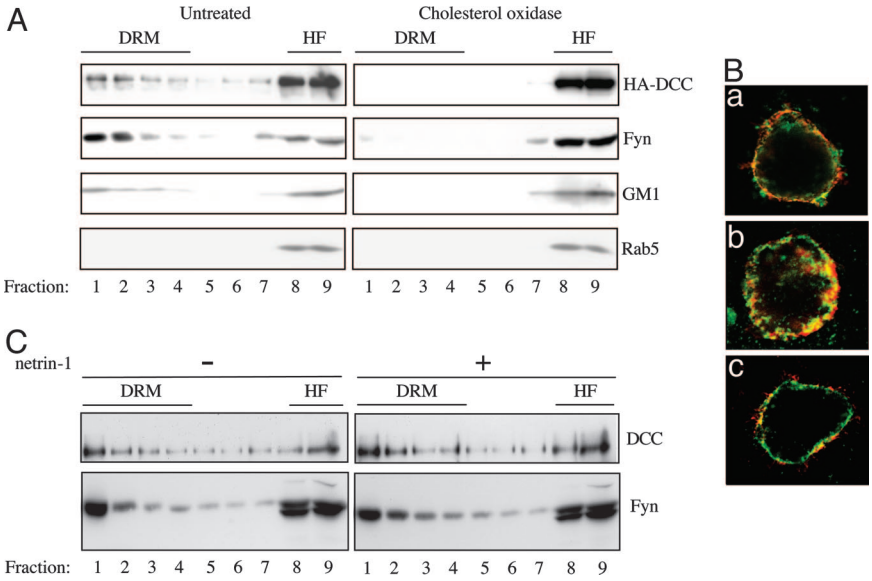

Fig. 1. DCC association in lipid membrane rafts. HF, heavy nonraft fraction; DRM, detergent-resistant membrane. (*A*) HEK293 T cells were transiently transfected with the hemagglutinin epitope (HA)-tagged DCC-expressing construct, and cell lysates were solubilized in Brij 98 and subjected to a sucrose gradient. Immunoblotting performed on the different sucrose fractions was visualized with horseradish peroxidase (HRP)-conjugated anti-HA (HA-DCC), Fyn, and Rab5 antibodies or with CTB-HRP (GM1). (*B*) Immunostaining on DCC (red) and GM1 (green) in the absence (*a*) or after (*b* and *c*) GM1 cross-linking by CTB/anti-CTB. Before staining, the cells were pretreated (*c*) or not (*b*) with CO (1 unit/ml). (*C*) The same procedure as in *A* but here HEK293 T cells were treated or not with 300 ng/ml netrin-1 24 h before collection. Note that the DRM localization of DCC is not significantly different in the presence or absence of netrin-1.

missural neuronal culture, most probably by allowing an optimal formation of the DCC-associated caspase-activating complex.

Results and Discussion

Lipid rafts can be isolated, thanks to their characteristic detergent-insolubility upon membrane solubilization in Brij 98, and separated from the disordered membrane environments by sucrose density gradient centrifugation. As shown in Fig. 1*A*, upon DCC expression in human embryonic kidney 293 (HEK293) T cells, a substantial proportion of DCC is found in the light fractions [detergent-resistant membranes (DRMs)] that contain the lipid raft and are also enriched in raft markers, such as Fyn and GM1 glycosphingolipid. Conversely, the nonraft marker rab5 was exclusively detected in heavy nonraft fractions (HFs). To further delineate the cholesterol dependence of the buoyant fractions, we tested the effect of cholesterol oxidase (CO), which converts cholesterol to cholestenone and therefore disorganizes the composition of the lipid microdomain. Preincubation of the cells with CO (Fig. 1*A*) abolished the presence of the Fyn raft marker and of DCC in the buoyant fractions. To further demonstrate the association of DCC with lipid rafts, we also performed fluorescence microscopy analysis in HEK293 T cells and analyzed the immunostaining of DCC before and after GM1 cross-linking induced by the cholera toxin subunit B (CTB)/anti-CTB. As seen in Fig. 1*B*, in unpatched conditions (*Top*) DCC and GM1 were evenly distributed, whereas the GM1-containing patches formed after cross-linking were highly enriched in DCC (*Middle*), a colocalization that was lost in cells pretreated with CO (*Bottom*). These results are consistent with the biochemical data and further demonstrate the association of DCC with membrane rafts. Interestingly, DCC presence in lipid rafts seems to be constitutive and is merely dependent on its ligand. Indeed, as shown in Fig. 1*C*, netrin-1 treatment failed to significantly modulate DCC presence in DRMs.

While looking for a functional explanation of the localization of DCC to rafts, it was of interest to note that receptors involved in cell death induction, such as Fas, TNFR1, and DR5, have been reported to be located in lipid rafts (20–23). Moreover, this localization seems to be a prerequisite for their ability to trigger cell death. We then investigated whether the ability of DCC to induce cell death could be modulated by raft disruption. To achieve this, HEK293 T cells were transiently forced to express DCC (Fig. 2*A*) in the presence or absence of CO treatment, and cell death was first measured by a trypan blue exclusion assay. As shown in Fig. 2 *B* and *C*, although DCC induces the death of HEK293 T cells in the absence of CO treatment, raft disruption was associated with a significant decrease in DCC-induced cell death. To further investigate the effect of raft disruption on DCC-induced apoptosis, apoptosis was quantitated by monitoring caspase activation, either by counting the cells stained with an active caspase-3 antibody or by measuring the ability of a cell lysate to cleave the DEVD-AFC substrate, releasing the fluorescent 7-amino-4-trifluoromethylcoumarin (AFC). As shown in Fig. 2 *D* and *E*, DCC-induced apoptosis was abrogated by CO treatment. A similar effect was observed when apoptosis was determined by a TUNEL technique (Fig. 2*F*). Because lipid rafts are enriched not only in cholesterol but also in sphingomyelin (SM), we tested DCC-induced cell death after SM depletion by using sphingomyelinase (SMase) pretreatment (24, 25). As shown in Fig. 2*E*, depletion of SM is associated with a similar inhibition of DCC-induced apoptosis. Moreover, lipid raft disorganization does not have a general inhibitory effect on apoptosis: The common apoptotic stimulus staurosporin induced apoptosis at a similar extent in the presence or absence of lipid raft integrity (Fig. 2*E*). Thus, DCC-induced cell death requires lipid raft integrity.

The finding that CO and SMase inhibit DCC-induced cell death addresses the question of the requirement of raft integrity for DCC-induced cell death, but it fails to discriminate between the effect of CO or SMase on raft integrity in general (e.g., a downstream effector of DCC-induced cell death needs to be localized at the raft) and the effect of these compounds on the specific presence of DCC in lipid rafts. We then took advantage of the palmitoylation-deficient mutant of DCC (DCC C1121V). Indeed, many raft-associated molecules have been shown to be palmitoylated (26, 27), and DCC has a conserved cysteine residue within its transmembrane domain that is indeed palmitoylated (19). Mutation of the transmembrane cysteine-1121 to valine decreased DCC local-

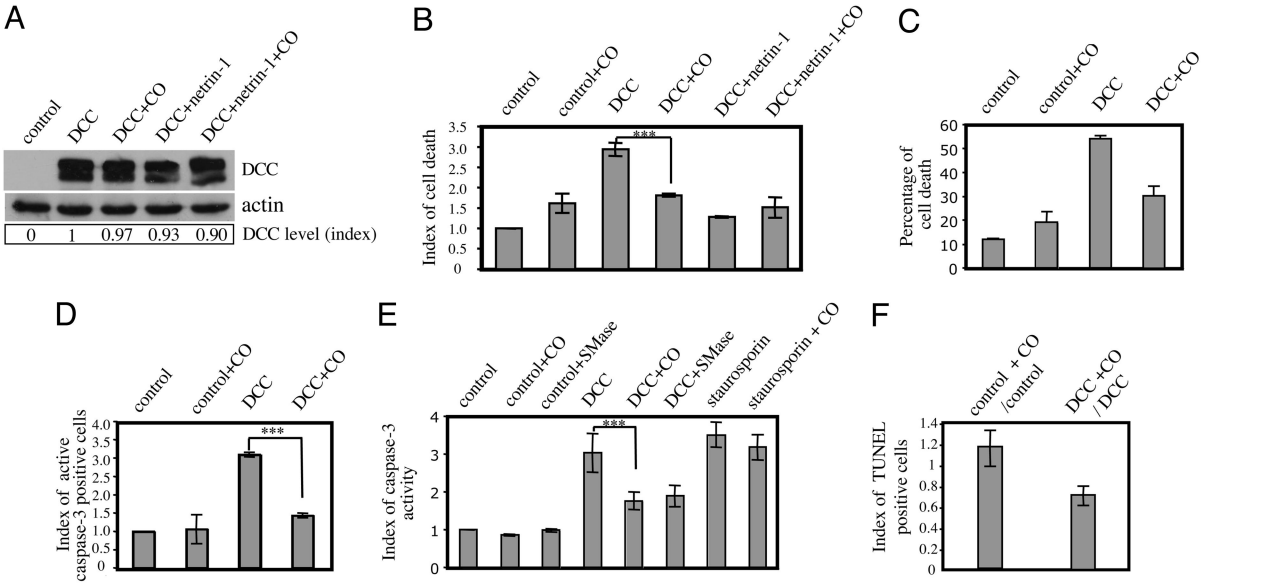

Fig. 2. DCC-induced cell death requires lipid raft integrity. HEK293 T cells were transiently transfected with the DCC-expressing construct (DCC) or a mock vector (control). CO, SMase, or netrin-1 treatment was performed 24 h after the beginning of the transfection, and cell death was measured 24 h later, as described in *Materials and Methods*. (*A*) Immunoblot showing DCC expression in the different tested conditions. A quantification of the immunoblot was performed by using NIH IMAGE software (http://rsb.info.nih.gov/nih-image) and is presented as an index of DCC level, with 1.0 being DCC expressed alone. (*B* and *C*) Cell death assay measured by trypan blue exclusion assay. (*B*) As described in *Materials and Methods*, an index of cell death is presented as the ratio between the percentage of cell death determined in each tested condition and the percentage of cell death obtained in the nontreated, mock-transfected cells. The histogram represents the average of six independent experiments. (*C*) One representative experiment is presented with the respective percentage of cell death. Standard deviations are indicated. (*D*) Apoptosis monitored by immunostaining with anti-active caspase-3. Immunostaining with anti-active caspase-3 was performed as described in *Materials and Methods*. An index of active caspase-3-positive cells is presented as the ratio between the percentage of active caspase-3-positive cells in the different tested conditions and the percentage of active caspase-3-positive cells in the nontreated, mock-transfected cells. The histogram presented is the average of three independent experiments. (*E*) Apoptosis measured by the ability of cell lysate to cleave the fluorescent caspase substrate DEVD-AFC. As described in *Materials and Methods*, equal amounts of lysates from 1×10^6 transfected and/or treated cells were incubated in the presence of DEVD-AFC, and fluorescence was determined by using a Victor station (Wallac, Gaithersburg, MD). An index of caspase-3 activity is presented as the ratio between the fluorescence obtained in the different tested conditions and the fluorescence measured in nontreated, mock-transfected cells. The histogram presented is the average of three independent experiments. Note that SMase treatment has an effect similar to that of CO. In the case of staurosporin treatment, 2 μM staurosporin was incubated for 12 h after CO treatment. (*F*) TUNEL staining was also performed as described in *Materials and Methods*. An index of TUNEL-positive cells is presented as the ratio between the percentage of TUNEL-stained cells in either the control or DCC-transfected population treated with CO and the percentage of TUNEL-positive cells in, respectively, mock- or DCC-transfected cells. The histogram presented is the average of three independent experiments. (*B*, *D*, and *E*) Student's *t* test; ***, $P < 0.01$.

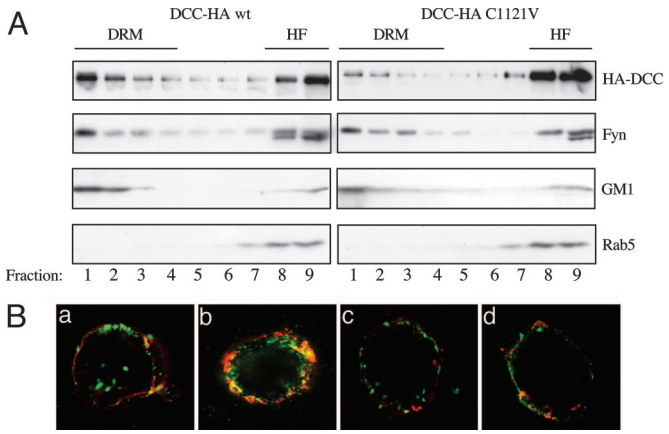

Fig. 3. Amino acid C1121 of DCC is crucial for DCC lipid raft localization. (*A*) HEK293 T cells were transfected with HA-tagged DCC or DCC C1121V-expressing constructs, and cell lysates were solubilized in Brij 98 and subjected to a sucrose gradient. Immunoblotting performed on the various sucrose fractions was visualized with HRP-conjugated anti-HA (HA-DCC), Fyn, and Rab5 antibodies or with CTB-HRP (GM1). wt, Wild type; HF, heavy nonraft fraction. (*B*) Immunostaining performed on DCC (red) wild-type (*a* and *b*) or C1121V (*c* and *d*) and GM1 (green) in the absence of (*a* and *c*) or after (*b* and *d*) GM1 cross-linking by CTB/anti-CTB. Note in *b* that the presence of yellow color representing colocalization is more prominent than that in *d*, where yellow can hardly be detected, thus suggesting that the DCC C1121V fails to colocalize with lipid rafts.

ization in the DRM fraction (Fig. 3*A*). Similarly, DCC C1121V failed to be copatched with GM1 in the immunostaining experiment (Fig. 3*B*). We then performed cell death assays, i.e., the trypan blue exclusion assay (Fig. 4*A*), or staining for active caspase-3 (Fig. 4*B*) on HEK293 T cells forced to express either wild-type DCC or the DCC C1121V mutant. Although DCC and DCC C1121V were expressed at similar levels (Fig. 4*C*), DCC C1121V failed to induce cell death. Taken together, these results demonstrate that DCC location in lipid rafts is a prerequisite for its ability to kill cells.

Because DCC is unable to trigger cell death when it is not localized to the raft, we analyzed whether the absence of DCC proapoptotic activity was associated with the lack of formation of the previously described DCC-associated caspase-activating complex (17). Specifically, it was shown that, in the absence of netrin-1, DCC was able to recruit caspase-9. We then checked the coimmunoprecipitation of DCC with caspase-9 in HEK293 T cells treated or not with CO. As shown in Fig. 5, in the absence of CO treatment, DCC clearly interacted with caspase-9. However, the DCC/caspase-9 interaction was strongly reduced after raft disruption. Moreover, to eliminate a potential side effect of CO, we investigated the interaction of caspase-9 with the DCC mutant, which fails to be lipid raft-located. As shown in Fig. 5, DCC C1121V failed to adequately interact with caspase-9. Thus, the presence of DCC in lipid rafts is probably required for the receptor's proapoptotic activity, because the lipid raft allows the adequate formation of the DCC-associated caspase-activating complex.

Looking for the biological relevance of these *in vitro* observations, we turned toward the most studied biological system involving

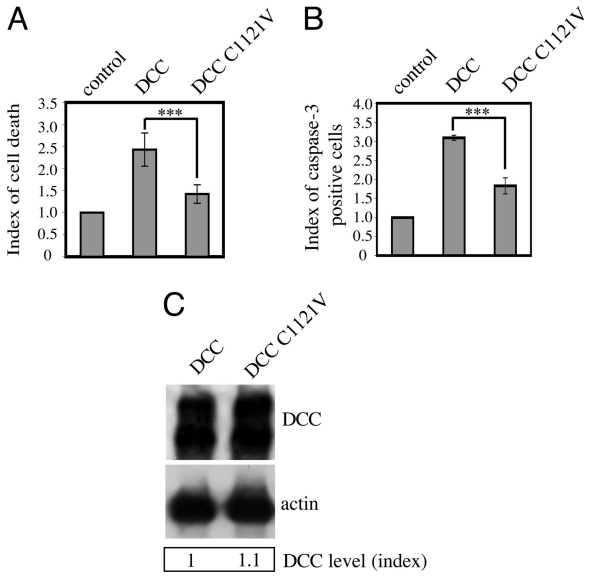

Fig. 4. The presence of DCC in the lipid raft is required for DCC proapoptotic activity. HEK293 T cells were transiently transfected with a DCC- or DCC C1121V-expressing construct (DCC or DCC C1121V, respectively) or a mock vector (control). Cell death was measured 48 h after the beginning of the transfection. (*A*) Cell death assay measured by trypan blue exclusion assay as shown in Fig. 2*B*. Index of cell death is presented as in Fig. 2*B*. (*B*) Apoptosis was measured as in Fig. 2*D* by counting cells stained with anti-active caspase-3. An index of cells stained with active caspase-3 is shown as in Fig. 2*D*. (*A* and *B*) The histograms presented are the average of three independent experiments. Standard deviations are indicated. Student's *t* test; ***, $P < 0.01$. (*C*) Immunoblot showing DCC and DCC C1121V expression. A quantification of the immunoblot was performed by using NIH IMAGE software and is presented as an index of DCC level, with 1.0 being DCC expressed alone.

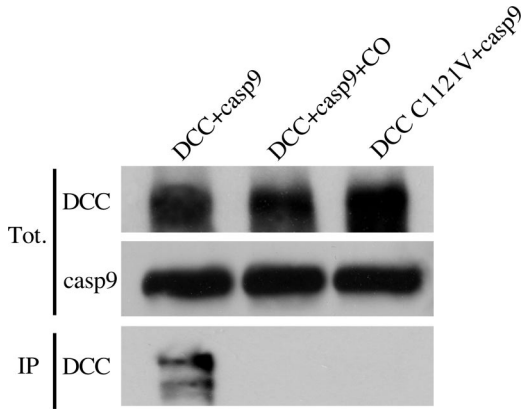

Fig. 5. DCC interaction with caspase-9 (casp9) is dependent on DCC localization in lipid rafts. HEK293 T cells were transiently cotransfected with the DCC- or DCC C1121V-expressing construct (DCC or DCC C1121V) together with HA-tagged catalytically inactive caspase-9 and treated or not with CO. The catalytically inactive mutant for caspase-9 was used rather than wild-type caspase-9 because caspase-9 overexpression is associated with massive cell death (17). Cells were subjected to immunoprecipitation 48 h after transfection by using anti-HA antibody for the pull-down. DCC/caspase-9 interaction was detected by an immunoblot probed with an anti-DCC antibody. Tot., DCC and caspase-9 ectopic expression before the pull-down; IP, DCC expression after the pull-down. Note that DCC pull-down by caspase-9 was detected much less when CO treatment was performed or when DCC C1121V was used instead of DCC wild type.

the DCC/netrin-1 pair. The DCC/netrin-1 pair has been mainly studied in developing commissural neurons. Commissural neurons that express DCC are known to extend axons from the dorsal spinal cord to the ventral spinal cord, as a result of the chemoattractivity of netrin-1 synthesized by the floor plate. We have suggested that netrin-1 could regulate not only axon guidance but also neuronal survival. Along this line, we have observed that commissural neurons from rat/mouse embryos [embryonic day 13 (E13)] cannot be cultured *in vitro* in the absence of netrin-1, as a result of massive cell death induction. This phenomenon is related to the intrinsic proapoptotic activity of DCC, because the cultured commissural neurons from DCC mutant embryos survive when grown in the absence of netrin-1 (unpublished work). We then assessed whether raft disruption provided a gain of survival similar to that due to the presence of netrin-1 in primary commissural neuronal culture. Dorsal spinal cord from E13 rat embryos was then dissected out, and primary commissural neurons were treated or not for 1 h with CO before being cultured for 24 h in the presence or absence of netrin-1. Cell death was then measured by TUNEL staining. As shown in Fig. 6, whereas all of the primary commissural neurons died in <24 h in the absence of netrin-1, the addition of netrin-1 was sufficient to allow survival of ≈50% of the cells. Interestingly, the addition of CO in the absence of netrin-1 yielded a similar survival effect, hence strongly suggesting that DCC-induced cell death is impaired in primary culture after raft disruption.

Here we have shown that, through palmitoylation, DCC partly localizes to lipid rafts, a prerequisite for its proapoptotic activity. This seems to be true not only with forced expression of DCC in immortalized transformed cells but also in primary neurons that endogenously express DCC. This finding recalls the trait of the so-called death receptors. It was indeed observed that Fas/CD95 needs to be located at the raft to respond to FasL and to trigger cell death (20, 28–30). Interestingly, Fas association with the lipid raft seems to be enhanced by T cell receptor (TCR) restimulation of activated human $CD4^+$ T cells (31), suggesting that, *in vivo*, the localization of death receptors within the lipid raft may be an important regulation means for the control of cell fate, i.e., death or survival. It is, however, unclear how Fas is recruited to the lipid raft. Regarding DCC, we have shown that DCC association is not dependent on ligand presence but is due to receptor palmitoylation (Fig. 1*C* and ref. 19). The nature of the mechanisms that regulate

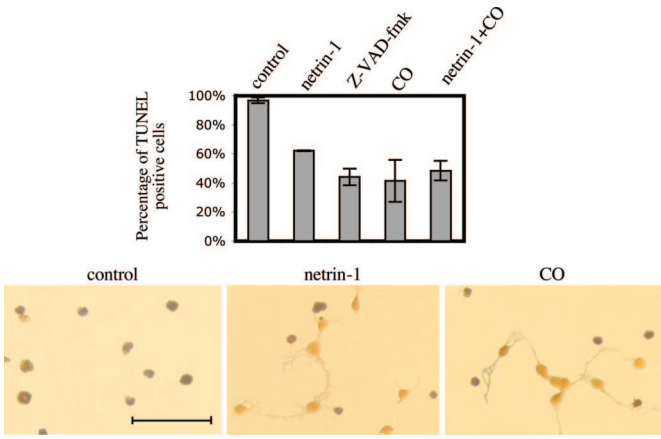

Fig. 6. DCC-mediated commissural neuronal apoptosis is dependent on lipid raft integrity. Commissural neurons from embryonic day 13 (E13) rat embryos were cultured in the presence or absence of netrin-1 for 24 h. The CO treatment was done for 1 h. Cell death was analyzed by TUNEL staining as described in *Materials and Methods*. (*Upper*) Histogram showing the percentage of TUNEL-positive cells. Standard deviations are indicated ($n = 3$). Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone, a pan-caspase inhibitor. Note that netrin-1 and CO treatments similarly allow the survival of ≈50% of the commissural neurons. (*Lower*) Representative TUNEL staining on commissural neurons grown in the absence of netrin-1 and CO (control) or in the presence of netrin-1 (netrin-1) or CO (CO). (Scale bar: 50 μm.)

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DCC palmitoylation is yet completely unknown and could represent an important line of investigation because of the suspected tumor suppressor activity of DCC. The actual model for the function of DCC as a tumor suppressor is related to its proapoptotic activity. It may then be a remarkably selective advantage for a tumor cell, on top of deleting a *DCC* allele by loss of heterozygosity (LOH) or inhibiting DCC expression by promoter methylation (for a review, see ref. 3), to block DCC proapoptotic activity, possibly by down-regulating the presence of the receptor in the lipid raft. Whether the presence of DCC in lipid rafts is modulated in tumors remains to be investigated.

An intriguing question, then, is why DCC fails to trigger apoptosis when located outside the lipid raft, even in the absence of netrin-1. The absence of Fas proapoptotic activity in the presence of FasL, which is observed after raft disruption, seems to be due to the inability of Fas to recruit important proteins from the death-inducible signaling complex (DISC), such as FADD or caspase-8 (20, 21). In the case of DCC, a similar mechanism seems to operate, because the described DCC-associated caspase-activating complex (17) fails to form adequately in the absence of lipid raft integrity, as shown here by the decreased interaction of DCC with caspase-9. This, however, does little to explain why DCC needs to recruit this caspase-activating complex within lipid rafts. Is it a way to select a more efficient killing mechanism with the adaptor and effector proteins already located in lipid rafts, or, on the contrary, is it a regulatory mechanism to avoid "leaky" death signaling outside lipid rafts that would be difficult to circumvent once caspases are activated? This question remains open.

Materials and Methods

Cells, Transfection Procedures, and Netrin-1 Production. Transient transfections of HEK293 T cells were performed as previously described (32). Briefly, HEK293 T cells were plated the day before transfection in either 35-mm dishes (1.5×10^5 cells per well; cell death assay, Western blot) or 100-mm dishes (1×10^6 cells per dish; immunoprecipitation). Transfections were then performed by using Lipofectamine for 6 h with a DNA-to-Lipofectamine ratio of 1 μg to 6 μl. HEK293 T cells were then grown for another 42 h in serum-plus medium (18 h) and serum-free medium (24 h), treated or not with lipid raft integrity modulators as described below, and treated or not with netrin-1 during the last 24 h of the transfection. Netrin-1 was purified from netrin-1-producing 293-EBNA cells according to Serafini *et al.* (33).

Immunoprecipitation and Western Blot Analyses. One-dimensional immunoblots using different commercially available antibodies raised against DCC, HA-DCC, Fyn, Rab5, and GM1 were performed as previously described (20, 32). HRP-coupled secondary antibodies were purchased from Jackson ImmunoResearch, and CTB-HRP was purchased from Sigma–Aldrich. Immunoprecipitations were carried out as described previously (17).

Plasmid Constructs. The full-length DCC-expressing construct pDCC-CMV-S, p-HA-DCC-CMV-S, or p-HA-DCC-C1121V-CMV-S, the Flag-tagged dominant negative mutant caspase-9-expressing construct have been described (8, 17, 19). The HA-tagged dominant negative caspase-9-expressing construct was obtained from the above-mentioned Flag-tagged construct by using the QuikChange (Stratagene) strategy.

Copatching Experiments. To analyze raft patching, DCC-transfected HEK293 T cells were first incubated with Alexa Fluor-conjugated CTB (Molecular Probes) for 45 min at 4°C, followed or not by anti-CTB antibody (Calbiochem) for 20 min at 37°C. After washing (three times) with PBS, cells were presaturated in PBS/2% BSA for 10 min and incubated consecutively with anti-DCC (Ab-1; Oncogene Research Products, San Diego) and a rhodamine-conjugated anti-mouse antibody. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min and mounted in moviol (Calbiochem). Confocal microscopy was performed with a Leitz DMBRE confocal microscope and ×100 objective lens.

Biochemical Raft Separation. Rafts were isolated as described in ref. 20. Briefly, postnuclear supernatant (PNS) from 3×10^7 HEK293 T cells was solubilized in 1 ml of buffer A (25 mM Hepes/150 mM NaCl/1 mM EGTA/protease inhibitors mixture) containing 1% Brij 98 for 5 min at 37°C and chilled on ice before being placed at the bottom of a step sucrose gradient (1.33, 0.9, 0.867, 0.833, 0.8, 0.767, 0.733, 0.7, to 0.6 M sucrose) in buffer A. Gradients were centrifuged at 38,000 rpm for 16 h in a SW41 rotor (Beckman Coulter) at 4°C, and 1-ml fractions were harvested from the top, except for the last one (no. 9), which contained 3 ml. The DRMs containing lipid rafts were fractions 1–4, and the heavy nonraft fraction (HF) consisted of pooled fractions 8 and 9.

Raft Disorganization. Transiently transfected HEK293 T cells were incubated in 37°C preheated serum-free 10 mM Hepes buffer containing 1 unit/ml CO or 1 unit/ml SMase at 37°C for 1 h. After treatment with CO or SMase, cells were washed once before raft isolation was performed or were cultured for another 24 h before cell death determination.

Cell Death Assays. HEK293 T cell death was analyzed by using the trypan blue staining procedure (17). Briefly, after 48 h of transfection, total cells (adherent and floating) were collected by mechanical detachment and medium collection. Cells were pelleted by slow centrifugation and resuspended in serum-free medium. Trypan blue (0.4%) was added in a ratio of 1:1 to the cell suspension, and the percentage of cell death was measured in a hemacytometer by counting, in a blinded fashion and within a given volume, the number of blue (dead) cells and comparing this number to the total number of cells in this same volume. Three independent series of cell counting were done for each sample with an average of 200 counted cells. An index of cell death was determined as the ratio between the percentage of cell death in the population transfected with the plasmid of interest and the percentage of cell death obtained in the mock-transfected population. Apoptosis was monitored by measuring caspase activation either by counting cells stained with anti-active caspase-3, as described in ref. 11, or by measuring caspase activity in cell lysate as described in ref. (8). Briefly, immunostaining with anti-active caspase-3 was performed by collecting transfected cells. Total (adherent and nonadherent) cells were collected, washed with PBS, and cytospun on glass. Cytospun cells were then fixed with 4% paraformaldehyde in PBS for 30 min, and permeabilization was performed by using 0.2% Triton X-100 in PBS for 30 min. The endogenous peroxidase activity was inhibited by preincubation in 5% H_2O_2 in PBS for 30 min. First antibody incubation was performed by using anti-active caspase-3 antibody (Cell Signaling Technology, Beverly, MA) followed by biotin-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). The peroxidase activity was then provided by a biotin/avidin/peroxidase complex (ABC kit, Vector Laboratories), and staining was performed by using 3,3′-diaminobenzidine chromogen. The percentage of caspase-3-stained cells was estimated by comparing the number of active caspase-3-stained cells with the total number of cells present on a given surface. An index is presented as the ratio between the percentage of stained cells in the population transfected with a plasmid of interest and the percentage obtained in the mock-transfected population. Caspase-3 activity was also measured by using the ApoAlert caspase-3 assays from Clontech. This assay utilizes the DEVD-AFC substrate. The activity was determined according to the manufacturer's instructions, and caspase activation is presented as the ratio between the caspase activity of the sample and that measured in HEK293 T cells transfected

with the mock vector. Apoptosis was also analyzed by counting cells stained by the TUNEL technique according to the manufacturer's instructions (Roche Diagnostics). Fixation, permeabilization, and staining were performed as before for caspase-3 staining except that the antibody reaction was replaced by cell incubation for 1 h at 37°C with terminal deoxynucleotidyltransferase and biotin-dUTP.

Primary Commissural Neuronal Culture. Primary commissural neurons were obtained from embryonic day 13 (E13) rat embryos by dissecting out the dorsal spinal cord as described elsewhere (34, 35). The tissues were then dissociated by using 5 mg/ml trypsin and 0.1 mg/ml DNase I (Sigma) in Hanks' balanced salt solution (HBSS) without calcium or magnesium (Invitrogen). The dissociated cells that were obtained were plated on poly(L-lysine)-precoated coverslips at 0.5×10^5 cells per well on a 24-well plate. Commissural neurons were then cultured over 24 h in neurobasal medium containing B27 supplement.

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